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Sir:

Transmitted herewith for filing is a provisional patent application under CFR 1.53(c) of Inventors:

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Title: SELECTIVE INCORPORATION OF 5-HYROXYTRYPTOPHAN INTO PROTEINS IN MAMMALIAN CELLS

Enclosed are:

[X] 35 pages of the application (including description, claims, abstract and appendix).
[X] 7 sheet(s) of [ ] formal [x] informal drawing(s).
[ ] Abstract.
[ ] _____ claims.
[ ] Small Entity Status is Claimed
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By: _____

Amelia Weintraub

Attorney Docket: 54A-001000US
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PROVISIONAL Patent Application For

SELECTIVE INCORPORATION OF 5-HYROXYTRYPTOPHAN INTO PROTEINS IN MAMMALIAN CELLS

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Abbreviations: aaRS, aminoacyl-tRNA synthetase; BsTrpRS, *B. subtilis* tryptopanyl-tRNA synthetase; tRNA, transfer RNA; mutRNA$^{Trp}_{UCA}$, mutant opal suppressor tRNA^{Trp}; 5-HTTP, 5-hydoxy-L-tryptophan; foldon, bacteriophage T4 fibritin foldon domain.

Abstract

An orthogonal tryptophanyl-tRNA synthetase (TrpRS) - opal suppressor mu $tRNA^{Trp}_{UCA}$ pair was generated for use in mammalian cells. The anticodon loop of the *Bacillus subtilis* $tRNA^{Trp}$ was mutated to UCA, three positions in the D-arm were mutated to generate an internal promoter sequence, and the $mutRNA^{Trp}_{UCA}$ gene was inserted between the 5' and 3' flanking sequences of the $tRNA^{Trp1}$ gene from *Arabidopsis* to enhance its expression in mammalian cells. *In vitro* aminoacylation assays and *in vivo* opal suppression assays showed that *B. subtilis* TrpRS (BsTrpRS) only charges the cognate mu $tRNA^{Trp}_{UCA}$ and no endogenous mammalian tRNAs. Similarly, the mu $tRNA^{Trp}_{UCA}$ is specifically charged by *B. subtilis* TrpRS and not by endogenous synthetases in mammalian cells. Site-directed mutagenesis was then used to alter the specificity of BsTrpRS to uniquely charge 5-hydoxy-*L*-tryptophan (5-HTTP). The resulting mutant BsTrpRS - $mutRNA^{Trp}_{UCA}$ pair allows the efficient and selective incorporation of 5-HTPP into mammalian proteins in response to the codon, TGA. This amino acid can be used as both a fluorescence probe and also undergoes electrochemical oxidation *in situ* to generate an efficient protein crosslinking agent.

Introduction

Recently, a general method was developed that makes possible the addition of new amino acid building blocks to the genetic codes of *Escherichia coli* (*E. coli*) (1) and *Saccharomyces cerevisiae* (*S. cerevisiae*) (2). In this approach, an orthogonal tRNA-aminoacyl tRNA synthetase pair is evolved which uniquely recognizes the amino acid of interest and selectively incorporates it into proteins in response to the amber nonsense codon, TAG. This methodology has been used to site-specifically incorporate a variety of unnatural amino acids into proteins with high fidelity and good efficiency, including amino acids with novel functional groups (3-6), photocrosslinkers (7, 8), heavy atoms, sugars (9) and redox active moieties (10). In addition, new orthogonal tRNA-synthetase pairs have been evolved from leucyl (11), lysyl, glutaminyl (12), aspartyl (13) and tyrosyl (14) tRNA-synthetase pairs in order to expand the number and structural diversity of amino acids that can be genetically encoded in bacteria and yeast.

In an effort to extend this methodology to mammalian cells, two general approaches are being developed. The first involves the directed evolution of an orthogonal tRNA-synthetase pair with a desired specificity in yeast, and the subsequent adaptation of this pair for expression in mammalian cells (2). This approach has the advantage that large libraries of tRNA synthetases can be generated in yeast and a mutant with the desired specificity can be efficiently isolated using an appropriate genetic selection or screen. Alternatively, one can use structure-based design to generate a mutant orthogonal tRNA-synthetase pair with altered specificity directly in mammalian cells. Recently, Yokoyama and coworkers used a variant of the former approach to generate a heterologous orthogonal pair consisting of a *Bacillus stearothermophilus* amber suppressor $tRNA^{Tyr}$

and mutant *E. coli* tyrosyl-tRNA synthetase that was able to incorporate 3-iodo-*L*-tyrosine into proteins in mammalian cells with 95% fidelity (15, 16). To further expand the number of unnatural amino acids that can be genetically encoded in mammalian systems, we now report the generation of an orthogonal mammalian tRNA-synthetase pair from a *Bacillus subtilis* (*B. subtilis*) tryptophanyl tRNA and cognate synthetase. Moreover, we show that directed mutagenesis of this pair can be used to generate a mutant synthetase that efficiently inserts 5-hydroxy-tryptophan (5-HTPP) into proteins in response to the opal codon TGA with excellent fidelity. This amino acid has novel spectroscopic and electrochemical properties that can be used to probe protein structure and function both *in vitro* and *in vivo*.

Materials and Methods

General. Mammalian cells were transfected with Fugene 6 reagent (Roche). Radio-labeled amino acids were obtained from Perkin Elmer (Boston, MA) and oligonucleotides were from Proligo (La Jolla, CA). Genomic DNAs were obtained from ATCC (Manassas, VA). Antibodies, antibiotics and TRIZOL solution were purchased from Invitrogen (Carlsbad, CA). V5-antibody-immobilized agarose was purchased from Bethyl Laboratories, Inc. (Montgomery, TX). 5-Hydroxy-*L*-tryptophan was from Sigma (St. Louis, MO) and used without further purification. Nucleobond columns were purchased from Clontech (Palo Alto, CA).

Strains. *E. coli* strains DH10B and TOP10 were used for plasmid propagation and isolation. Human kidney 293T cells were used for unnatural amino acid incorporation into proteins.

Plasmids. The DNA fragment encoding *B. subtilis* TrpRS (BsTrpRS) was amplified from genomic DNA by polymerase chain reaction (PCR) and cloned into the *Xho*I-*Pac*I sites of the pMH4 vector (GNF, La Jolla, CA). The resulting plasmid pMHTrpRS encodes BsTrpRS with a His6 tag at the N-terminus in *E. coli*. To express BsTrpRS in mammalian cells, the PCR fragment encoding the synthetase was ligated into a pEF6-V5-His6-TOPO vector (Invitrogen, Carlsbad, CA). The resulting plasmid pEF6-TrpRS encodes wild-type *B. subtilis* TrpRS with C-terminal V5 and His6 epitope tags. A series of mutant synthetases was generated in this vector by site-directed mutagenesis using QuikchangeXL (Stratagene, La Jolla, CA) and mutagenic primers.

The suppressor mutRNA$^{Trp}_{UCA}$ gene was constructed by annealing two oligodeoxynucleotides. The first encodes the corresponding mutRNA$^{Trp}_{UCA}$ sequence fused to the 5'-flanking sequence (TAAAATTAATTAAACGTTTAGAAATATATAGATGAACTTTATAGTACAA) of the tRNA^{Trp1} gene (17). The second oligonucleotide consists of the corresponding mu tRNA$^{Trp}_{UCA}$ fused to the 3'-flanking sequence GTCCTTTTTTTG (17). Klenow was used to generate a duplex DNA which was inserted into the *Pst*I and *Xho*I sites of pZeoSV2(+) (Invitrogen, Carlsbad, CA). The resulting plasmid pTrptRNA can be used to transcribe mu tRNA$^{Trp}_{UCA}$ in mammalian cells.

The plasmid pFoldon which was used to express the bacteriophage T4 fibritin foldon domain (18) in 293T cells was constructed by inserting the PCR-amplified gene fragment into the pCDA3.1-V5-His-TOPO vector (Invitrogen, Carlsbad, CA). pFoldonTGA, which encodes the Trp68TGA foldon mutant, was constructed by site-

directed mutagenesis using the QuikchangeXL method and the corresponding HPLC-purified primers.

Expression and detection of mu $tRNA^{Trp}_{UCA}$ in mammalian cells. Mammalian 293T cells were transfected with plasmid pTrptRNA and incubated at 37°C under 5% CO_2 for 60 hours. Cellular RNA was extracted with TRIZOL solution according to manufacturer's instructions (Invitrogen) and the total tRNA was then isolated using a NucleoBond column according to manufacturer's protocol (Clontech). The yield and purity of the purified tRNA were analyzed with a 3% agarose gel. To detect the mu $tRNA^{Trp}_{UCA}$, the purified tRNAs were first blotted and then cross-linked onto nylon transfer membranes (Osmonics, Westborough, MA) by UV irradiation using Stratalinker 2400 (Stratagene) for 1 min. Following irradiation, the membrane was incubated in 100 ml of hybridization buffer (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0, 1% SDS, 5X Denhardt's reagent with 25 μg/ml sperm whale DNA) and gently shaken at 68°C for 1 hour. The oligonucleotide, CGGAGGTTTTGAAGACCTCTGCT, which is complementary to nucleotides 27 to 44 of the suppressor tRNA, was 5'-labeled with [γ-^{32}P]ATP and used to probe the membrane at 50°C for 6 hours. The membrane was then washed three times with wash buffer (15 mM NaCl, 1.5 mM sodium, pH 7.0, 0.1% SDS). The intensity of each dot was quantified using a PhosphorImager (Molecular Dynamics).

Expression of *B. subtilis* TrpRS in mammalian 293T cells. Cells were transfected with the plasmid pEF6-TrpRS and incubated at 37°C under 5% CO_2 for 60 hours. Cells were harvested and lysed with 1X passive lysis buffer (Promega, Madison, WI), and the cell lysate was centrifuged at 20,000 ×*g*. Proteins were separated by denaturing SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane.

Proteins were probed with primary anti-His6 antibody followed by secondary horseradish peroxidase-conjugated goat anti-rabbit IgG. Substrate (SuperSignal West Dura, Pierce) was applied to visualize the signals.

***In vitro* aminoacylation assay.** Aminoacylation assays were performed by methods described previously (19) in 20 μL reactions containing 50 mM Tris-HCl, pH 7.5, 30 mM KCl, 20 mM $MgCl_2$, 3 mM glutathione, 0.1 mg/ml BSA, 10 mM ATP, 1 μM (33 Ci/mmol) *L*-[5-^{3}H]-tryptophan, 750 nM synthetase, and 20 μM purified total tRNA. Assays were carried out to 10% conversion.

Opal suppression in mammalian cells. Transfections were carried out with Fugene 6 using a total of 2 μg DNA per 9.5 cm^2 plate according to the manufacturer's protocol (Roche). Minimum essential alpha medium (Gibco BRL) was used as the growth medium. Cell extracts were prepared 48 hours after transfection and subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blot using anti-V5 antibody (Invitrogen) and the SuperSignal West Dura immunodetection system (Pierce). The signals were detected by exposing the membrane to Hyperfilm MP (Amersham Pharmacia).

Unnatural amino acid incorporation in mammalian cells. Mammalian 293T cells were co-transfected with plasmids pTrptRNA, pFoldonTGA and individual mutant pEF6-TrpRS as previously described. After 24 hours, the culture medium was changed to minimum essential alpha medium containing 1 mM 5-hydroxy-*L*-tryptophan and appropriate antibiotics. After an additional 48 hours at 37°C under 5% CO_2, cells were harvested, lysed with 1X passive lysis buffer (Promega, Madison, WI), and the cell lysate was collected by centrifugation at 20,000 $\times g$. The foldon protein containing 5-hydroxy-

L-tryptophan was purified from the cell lysate (twenty 50 ml culture plates) with Ni-NTA beads followed by anti-V5-immobilized agarose beads according to manufacturer's protocol (Bethyl Laboratories, Montgomery, TX). An aliquot of the purified protein was subjected to high resolution electrospray ionization mass spectrometry.

Fluorescence spectroscopy. Proteins were diluted to a final concentration of 50 nM in 10 mM K_2PO_4, 100 mM KCl buffer at pH 7.5. Fluorescence spectra were measured on a Fluromax-2 spectrofluorimeter and corrected. Excitation spectra were recorded with an excitation bandpass of 4 nm and an emission bandpass of 8 nm; emission spectra were recorded with emission bandpass of 4 nm.

Electrochemical characterization of proteins containing 5-hydroxy-*L*-tryptophan. A conventional three-electrode cell, consisting of a gold electrode, a glassy carbon auxiliary electrode isolated by a glass frit, and a saturated calomel electrode (SCE) connected to the working volume with a Luggin capillary, was used for electrochemical measurements. The cell was placed in a grounded Faraday cage. Cyclic voltammetry measurements were performed using a potentiostat (Princeton Applied Research, model VMP2, Oak Ridge, TN) connected to network operated software EC-Lab v6.61. All electrochemical measurements were performed in 0.1 M phosphate buffer, pH 7.4 under argon atmosphere. Substrate 5-HTPP was dissolved in 100 mM phosphate buffer to a final concentration of 10 μg/mL. Potentials were measured in the range of 0-800 mV at a scan rate of 1 $V \cdot sec^{-1}$. For crosslinking experiments, the electrode potential was set to 800 mV for 30 minutes in the presence of 10 μg/mL wild type foldon or 5-HTPP-foldon protein, 0.1 M phosphate buffer, pH 7.4 under argon atmosphere. After that, the

solutions were collected, proteins were desalted by dialysis, concentrated and loaded on a gel for further analysis.

Results and Discussion

An orthogonal opal suppressor tRNA for use in mammalian cells. To genetically encode an unnatural amino acid in mammalian cells, one must generate an orthogonal tRNA which is not recognized by any of the endogenous aminoacyl tRNA synthetases and at the same time efficiently incorporates its cognate amino acid in response to a unique codon, in this case the opal nonsense codon TGA. A corresponding aminoacyl-tRNA synthetase is also required which uniquely recognizes this tRNA and selectively charges it with the unnatural amino acid, and no endogenous amino acids. One approach to the generation of orthogonal tRNA-synthetase pairs takes advantage of inter-species differences in tRNA recognition elements (20). For example, Xue and coworkers have shown that *B. subtilis* $tRNA^{Trp}$ is not a substrate for the tryptophan-tRNA synthetases from yeast and mammalian cells (21). In addition, kinetic studies further demonstrated that mutation of the anticodon loop of this tRNA has only a minor effect (< 5%) on aminoacylation by the cognate *B. subtilis* TrpRS (22). Thus *B. subtilis* $tRNA^{Trp}$ is a likely candidate for an orthogonal suppressor tRNA in the mammalian cells.

Unfortunately when we tried to express *B. subtilis* $tRNA^{Trp}$ in 293T cells, no transcribed RNA was observed based on Northern Blot analysis of isolated total tRNA (*vide infra*) (23). Therefore, a series of modifications were made to the *B. subtilis* suppressor $tRNA^{Trp}$ (Fig. 1). Transfer RNAs in eukaryotes are transcribed by RNA polymerase III which recognizes two conserved intragenic transcriptional control

elements, the A box and the B box (24). Since the *B. subtilis* $tRNA^{Trp}$ sequence contains only the B box, nucleotides A7, A9, U11 were changed to G7, G9 and C11 in order to generate a pseudo A box, and the resulting mismatched base pairs G7-U64 and C11-A23 were replaced with G7-C64 and C11-G23, respectively. *In vitro* kinetic data showed that the A9G and U11C mutations have little effect on *B. subtilis* TrpRS recognition (22). Expression of the $tRNA^{Trp}$ gene in eukaryotes also depends upon 5' flanking sequences which are distinctly AT rich and contain several possible TATA elements (17). Therefore, we added the 5' flanking sequence of the $tRNA^{Trp1}$ gene from *Arabidopsis* (*Trp1*) which was previously shown to enhance the transcription of the plant $tRNA^{Trp}$ gene in human 293T cells (17). Since a properly positioned terminator element is the only 3' flanking sequence required for efficient expression of the plant $tRNA^{Trp}$ gene, the natural 3' flanking sequence of the same $tRNA^{Trp1}$ gene was used. Finally, the trinucleotide anticodon sequence CCA was changed to the opal suppressor UCA.

Figure 1.

The expression of the modified opal suppressor $tRNA^{Trp}$ ($mutRNA^{Trp}_{UCA}$) was verified using a Northern blot assay. The mutant $tRNA^{Trp}_{UCA}$ gene together with its 5' and 3' flanking sequences were cloned into the mammalian vector pZeoSV2(+) and the resulting plamid was transfected into human 293T cells using Fugene 6. Total tRNA was then isolated and blotted onto a membrane. As a control, the same amount of total tRNA from human 293T cells, beef liver and *E. coli* was also transferred onto the same membrane (Figure 2A). A synthetic oligonucleotide complementary to nucleotides 27 to 44 of the $mutRNA^{Trp}_{UCA}$ and labeled with [γ-^{32}P]ATP was used as a probe for the mut $tRNA^{Trp}_{UCA}$. Only the total tRNA isolated from transfected 293T cells produced a

signal (lane 4, Figure 2B); the control tRNAs gave no signal when incubated with the radioactive oligonucleotide probe (lane 1-3, Figure 2B). These results demonstrate that the $mutRNA^{Trp}_{UCA}$ is expressed in mammalian cells.

Figure 2.

BsTrpRS is an orthogonal synthetase in mammalian cells. Given the availability of an orthogonal mammalian suppressor tRNA, we next examined whether the corresponding BsTrpRS efficiently aminoacylates the $mutRNA^{Trp}_{UCA}$ and not the endogenous mammalian tRNAs. To determine the efficiency of aminoacylation of $mutRNA^{Trp}_{UCA}$ by BsTrpRS, *in vitro* aminoacylation assays were carried out with BsTrpRS purified from *E. coli*. Plasmid pMHTrpRS was used to express BsTrpRS with an N-terminal His6 tag, under control of an *L*-arabinose promoter. BsTrpRS was purified by Ni-NTA affinity chromatography with a yield of 5 mg/L. *In vitro* aminoacylation assays were then performed with ^{3}H-labeled tryptophan and various total tRNAs. BsTrpRS was found to efficiently charge the total tRNA isolated from *B. subtilis* cells containing cognate *B. subtilis* $tRNA^{Trp}$. In agreement with the published data (22), BsTrpRS did not aminoacylate total mammalian tRNA isolated from 293T cells to detectable levels. However, total tRNA isolated from transfected 293T cells expressing mu $tRNA^{Trp}_{UCA}$ was efficiently charged with ^{3}H-tryptophan by BsTrpRS. The overall aminoacylation activity of BsTrpRS for $mutRNA^{Trp}_{UCA}$ in mammalian total tRNA is about 40% of that for *B. subtilis* $tRNA^{Trp}$ in total bacterial tRNA, probably due to the lower expression level of mu $tRNA^{Trp}_{UCA}$ in mammalian cells. Nevertheless, this experiment

suggests that BsTrpRS can efficiently charge mutRNA$^{Trp}_{UCA}$, and, importantly, does not aminoacylate endogenous mammalian tRNAs to any appreciable extent.

BsTrpRS was expressed in mammalian cells using plasmid pEF6-TrpRS, which carries the BsTrpRS gene with a C-terminal His6 tag under the control of the human promoter EF-1α. Mammalian 293T cells were transiently transfected with plasmid pEF6-TrpRS using Fugene 6. Protein from the cell lysate was separated by SDS-PAGE, and subjected to a Western blot assay which was probed with anti-C-terminal V5 antibody. A band corresponding to the full length prokaryotic BsTrpRS protein (~36 kDa) was observed, demonstrating that the synthetase can be expressed in mammalian cells at reasonable levels (lane 1, Figure 4). No significant effect on growth rates was observed upon expression of the exogenous *B. subtilis* TrpRS.

Opal suppression in 293T cells is dependent on the expression of the BsTrpRS-mu tRNA$^{Trp}_{UCA}$ pair. We next determined the ability of the mutRNA$^{Trp}_{UCA}$-BsTrpRS pair to efficiently suppress an opal mutation in mammalian cells. To this end, the codon for Trp68 in a modified bacteriophage T4 fibritin *foldon* gene under control of a CMV promotor (18) was mutated to the opal codon, TGA. Based on previous data (25), mutation of Trp68, which is located in the interior of the foldon protein, to a tryptophan analogue is unlikely to disrupt the structure of this protein. To detect the expression of the full-length foldon protein, a V5 epitope tag and a His6 tag were fused to the C-termini of the wild type (pFoldonWT) and mutant proteins (pFoldonTGA). These corresponding *foldon* genes were transfected into human 293T cells along with either one or both of the BsTrpRS and mutRNA$^{Trp}_{UCA}$ genes. Full length protein was detected by a Western blot of the cell extracts with an anti-V5 antibody.

Figure 3.

No full-length protein was expressed when 293T cells were transfected only with either the mutant *foldon* gene (pFoldonTGA) (lane 1, Figure 3), or the mutant *foldon* gene and wild type BsTrpRS (lane 2, Figure 3). These results show that human 293T cells do not contain intrinsic opal suppressors of the TGA68 mutation. Suppression of the opal mutation was also not observed in the absence of wild type BsTrpRS and in the presence of mutRNA$^{Trp}_{UCA}$ (lane 3, Figure 3), confirming that the mutRNA$^{Trp}_{UCA}$ is not charged by endogenous synthetases in human 293T cells. In contrast, in the presence of the mutRNA$^{Trp}_{UCA}$, wild type BsTrpRS and TGA68 mutant *foldon* gene, expression of the full-length protein was detected (lane 4, Figure 3). For comparison, lane 5 shows the expression of wild type (wt) foldon protein in 293T cells. Based on the band intensities (lane 4 and 5, Figure 3), the suppression efficiency is approximately 38%. These experiments, together with the above *in vitro* aminoacylation assays, show that BsTrpRS aminoacylates only mutRNA$^{Trp}_{UCA}$ and not other endogenous mammalian tRNAs, and that the expressed mutRNA$^{Trp}_{UCA}$ is charged only by its cognate BsTrpRS and not by other endogenous mammalian synthetases. Thus, *B. subtilis* TrpRS-mutRNA$^{Trp}_{UCA}$ represent an orthogonal pair for use in mammalian cells.

The suppression efficiency of this cognate pair of tRNA^{Trp}-TrpRS is significantly higher than that of the reported heterologous pair of tRNA^{Tyr}-TyrRS in mammalian cells (16) and similar to the efficiencies for the human suppressor tRNA^{Tyr} and other suppressor tRNAs functioning in mammalian cells (20-40%) (26-28). Yokoyama *et al.* have shown that a gene cluster of nine copies of a suppressor tRNA can significantly increase its suppression efficiency in mammalian cells (16). We have not attempted to

use this method since a single copy of $mutRNA^{Trp}_{UCA}$ gene is sufficient to suppress the TGA68 codon and produce full-length protein at a level that can be detected by Western blot (≥ 10 pg/cell). In addition, toxicity was observed when the level of $mutRNA^{Trp}_{UCA}$ gene expression was increased by transfecting 293T cells with 4μg versus 2μg plasmid pTrptRNA/10^6 cells.

Site-specific incorporation of 5-hydroxy-*L*-trytophan (5-HTPP) into mammalian cells. We next asked whether the orthogonal $mutRNA^{Trp}_{UCA}$ - BsTrpRS pair could be used to selectively incorporate 5-hydroxy-tryptophan (5-HTPP) into proteins in mammalian cells in response to the opal nonsense codon. This amino acid has unique spectroscopic and redox properties that can serve as useful probes of protein structure and function both *in vitro* and *in vivo*. It is known that wild type *B. subtilis* TrpRS does not utilize 5-HTTP as a substrate (29). Therefore, in order to use BsTrpRS to selectively incorporate 5-HTPP into proteins, the active site of the synthetase must be mutated to charge 5-HTPP and not tryptophan. Although the structure of BsTrpRS has not yet been solved, the structure of a highly homologous tryptophanyl-tRNA synthetase (from *Bacillus stearothermophilus)* has been solved to 1.9 Å resolution (30-32). In this enzyme, the active site has a figure eight like shape with two adjacent binding pockets separated by an α-helix peptide consisting of residues Asp140, Ile141, Val142, Pro143, Val144, and Gly145. Val144 points directly towards C5 of tryptophan, providing unfavorable steric interactions with any tryptophan analogue containing a substituent at the 5 position. Mutation of Val144 to a smaller amino acid might therefore provide space for 5-substituted tryptophan analogues.

To test this notion, Val144 of wild type BsTrpRS was mutated to each of the other nineteen amino acids by site-directed mutagenesis and each mutant was assayed for its ability to aminoacylate $mutRNA^{Trp}_{UCA}$ with 5-HTPP by suppressing the TGA68 in the mutant *foldon* gene. The transfected cells were then grown in the presence or absence of 1 mM 5-HTPP and full-length protein was detected by Western blot of the cell extracts with an anti-V5 antibody (Figure 4). Expression of a full-length foldon protein in the presence of 5-HTPP would indicate that either 5-HTPP or a natural amino acid (likely tryptophan) is incorporated at position 68 of the foldon protein. One can exclude the incorporation of a natural amino acid by showing that no full length protein is expressed in the absence of 5-HTPP under otherwise the same conditions. Among the 19 TrpRS mutants, the Val144Gly mutant was able to suppress the TGA68 codon in the presence of 1 mM 5-HTPP and $mutRNA^{Trp}_{UCA}$. However, in the absence of 5-HTPP, the mutant BsTrpRS and $mutRNA^{Trp}_{UCA}$ were still able to suppress the opal mutation, indicating the Val144GlyBsTrpRS mutant also charges a natural amino acid. Only one other TrpRS mutant, Val144ProBsTrpRS, was able to suppress the TGA68 mutation in the presence of 1 mM 5-HTPP and $mutRNA^{Trp}_{UCA}$ (lane 5, Figure 4). Moreover, human 293T cells containing the Val144ProBSTrpRS and the TGA68 *foldon* gene were unable to produce full-length protein in the absence of either 5-HTPP or $mutRNA^{Trp}_{UCA}$ (lane 2-4, Figure 4). These results show that the Val144ProBsTrpRS mutant selectively aminoacylates the $mutRNA^{Trp}_{UCA}$ with 5-HTPP, and not with any endogenous amino acids. The yield of the HTPP68 mutant protein is approximately 100 μg/liter of culture, compared to that of about 1 mg/liter for wt protein.

Figure 4.

In order to confirm that the expressed mutant protein contains 5-HTPP, the protein was purified first by Ni-NTA affinity chromatography and, subsequently, by immuno-precipitation using anti-V5-immobilized agarose beads. An aliquot of the purified protein was subjected to high resolution electrospray ionization (ESI) mass spectrometry. The calculated molecular weight of the HTPP68 mutant protein is 14323.6 Da; the observed molecular weight is 14323.69 Da. No peak corresponding to wild type foldon protein was observed. This result clearly demonstrates that 5-HTPP is incorporated with high fidelity (>97%) into protein in response to the opal codon in mammalian cells.

It is somewhat surprising that a single mutation at the active site of BsTrpRS completely alters its specificity from *L*-tryptophan to 5-HTPP. Although the X-ray crystal structure is not yet available, computer-assisted modeling using Macromodel (version 8.1, Schrodinger, LLC.) suggests that the Val144Pro mutation generates space for the indole ring to rotate and abolishes an indole NH-Asp hydrogen bond. This may explain why the Val144ProBsTrpRS does not charge *L*-tryptophan. However, new hydrogen bonds are formed in the case of 5-HTPP with the 5-OH group hydrogen bonding with the imidazole side chain of His44 and the carboxylate group of Asp133, and the indole NH hydrogen bonding with the hydroxyl group of Ser7 (Figure 5).

Figure 5.

We are currently generating a library of BsTrpRS variants with several other sites randomly mutated, in order to identify mutants that selectively recognize additional side chain structures.

5-HTPP as a probe for protein structure and function. 5-Hydroxy-*L*-tryptophan has significant absorbance at 310 nm at pH 7.5 (ε=2450 $M^{-1}cm^{-1}$) (29), compared to that of tryptophan (ε=62 $M^{-1}cm^{-1}$) at 310 nm (36), suggesting 5-HTPP may be a usefil spectroscopic probe in proteins. Wild type foldon protein has only one tryptophan residue which is substituted in the mutant foldon protein with 5-HTPP. To compare the fluorescence properties of these two proteins, they were purified and then excited at 310 nm at pH 7.4, and their emission spectra were recorded (Figure 6). The HTPP68 foldon protein has an emission maximum, λ_{max}, at 334 nm, while the wild type foldon protein has a fluorescence λ_{max} at 367 nm. When both proteins are excited at 310 nm, the magnitude of fluorescence emission at 334 nm from HTPP68foldon protein is 11 times higher than that from wild type foldon protein. Such spectral shifts may make 5-HTPP a useful optical probe for some applications (29).

Figure 6.

5-HTPP can also undergo redox chemistry to afford tryptophan-4,5-dione (33). Cyclic voltammetry was used to determine whether the redox wave of 5-HTPP could be observed in the HTPP68foldon mutant. The voltammetric responses were measured for solutions containing 10 μM of HTPP, wt foldon or the foldon mutant. An anodic current originating from HTPP oxidation appears only in the presence of the mutant foldon or in a solution of free 5-HTPP with E = 400 mV and E = 450 mV, respectively, indicating the presence of 5-HTPP in the mutant foldon. The slight decrease in the oxidation potential for the mutant protein probably results from differential stabilization of the oxidized and reduced forms of 5-HTPP in aqueous solution versus the hydrophobic protein core

(34,35). No current was observed upon attempts to oxidize the wild type foldon (data not shown).

Upon electrochemical oxidation of 5-HTPP at a potential 800 mV in 7.4 phosphate buffer, the dimer (**1**, Figure 7A) is formed (33). Similarly, 5-HTPP can be oxidatively crosslinked to glutathione via its cysteine residue (**2**, Figure 7A). Therefore a 5-HTPP residue incorporated selectively into a protein might be useful as a redox cross-linker. In order to test this notion, we attempted to cross-link the HTPP68foldon mutant electrochemically by applying a positive potential of 800 mV to the working electrode in a solution containing either the HTPP68foldon protein or wild type foldon for 30 minutes in phosphate buffer. The resulting proteins were desalted, concentrated, denatured and separated using 4-20% gradient denaturing SDS-PAGE. The resulting gel was commassie-stained (Figure 7b). Lane 1 is the full-length HTPP68foldon mutant with a molecular weight of 14.5 kDa. Lane 3 is wild type foldon protein with the same apparent molecular mass. Lane 2 is the electrochemically oxidized product of the HTPP68foldon protein, which has a molecular weight of about 29 kDa, and corresponds to the dimeric mutant foldon protein. The yield is estimated to be 80% as determined from band intensities. In contrast, there is no cross-linked product in lane 4 which contains the oxidized wild type foldon protein under the same conditions. This result clearly shows protein cross-linking by the incorporated 5-HTPP. The exact mechanism of the protein cross-linking mediated by 5-HTPP is not yet clear and is under ongoing investigation.

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Figure legend

Figure 1: Cloverleaf structure of the *B. subtilis* tryptophan opal suppressor tRNA. The arrows indicate the sites of mutations. The solid box indicates the CCA sequence deleted in mu $tRNA^{Trp}_{UCA}$ gene.

Figure 2: Expression and Northern blot analysis of mu $tRNA^{Trp}_{UCA}$ obtained from 293T cells transfected with pTrptRNA. (A) Total tRNA was isolated from *E. coli* (lane1), beef liver (lane2), 293T cells (lane3) and 293T cells transfected with pTrptRNA plasmid (lane4), purified to homogeneity, and analyzed by electrophoresis on a 3% agarose gel. (B) The purified tRNAs from *E. coli* (lane1), beef liver (lane2), 293T cells (lane3) and 293T cells transfected with pTrptRNA plasmid (lane4) were blotted onto a membrane separately and probed with a 5'-^{32}P-labeled oligonucleotide complementary to nucleotides 27 to 44 of the mu $tRNA^{Trp}_{UCA}$.

Figure 3: Detection of opal suppression in 293T cells. The TGA68*foldon* gene (lane 1) and wild type *foldon* gene (lane 5), each with a V5 tag, were introduced into 293T cells. In the absence of either opal suppressor $tRNA^{Trp}$ (lane 2) or BsTrpRS (lane 3), no full-length protein was expressed as detected by western blot with anti-V5 antibody. In the presence of both opal suppressor $tRNA^{Trp}$ and BsTrpRS, the opal codon in the TGA68*foldon* gene was suppressed and the full-length *foldon* protein was expressed (lane 4).

Figure 4: Incorporation of 5-HTPP into foldon protein in 293T cells. The wild type BsTrpRS with a V5 tag was expressed in 293T cells (lane 1). In the absence of either 5-HTPP, $mutRNA^{Trp}_{UCA}$, or Val144ProBsTrpRS, no full-length protein was produced (lanes 2-4). In the presence of 5-HTPP, Val144ProBsTrpRS and $mutRNA^{Trp}_{UCA}$, the full-length foldon protein was expressed as detected by western analysis with anti-V5 antibody (lane 5).

Figure 5: Computational simulation of the complex between TrpRS and its substrates using Macromodel (Version 8.1, Schrodinger, LLC.). Hydrogen bonds are indicated as dotted lines (-------). The left structure illustrates the binding of wt *B. subtilis* TrpRS with its cognate substrate, tryptophan-5'AMP, including the hydrogen bond between the indole NH group and Asp133. The right structure illustrates the complex between the Val144ProBsTrpRS and its substrate, 5-HTPP-5'AMP. Note the disappearance of the hydrogen bond between the indole NH group and Asp 133, and the new hydrogen bonds between the 5-OH and His44, Asp133, and the indole NH and Ser7.

Figure 6: Fluorescence spetra of wt foldon protein (—) and the HTPP68 mutant protein (·····) with excitation at 310 nm.

Figure 7: Electrochemical protein crosslinking. (7a) **1.** product for dimerization of oxidized 5-HTPP molecules; **2.** product for reactionof oxidized 5-HTPP and cysteine; (7b) Oxidative crosslinking of proteins mediated by 5-HTPP. The proteins were separated with 4-20% gradient SDS-PAGE and commassie-stained. Lane 1 and lane 3

contain the purified HTPP68foldon and wild type foldon proteins, respectively. Lane 2 contains the crosslinked product for HTPP68 foldon, and lane4 contains the crosslinked product for wild type foldon protein. There is no crosslinked product for wild type foldon which has a monomeric molecular weight of 14.5 kDa. HTPP68foldon is crosslinked to afford a dimeric 29 kDa protein.

The following references are also considered with reference to this invention:

WO02/085932 "IN VIVO INCORPORATION OF UNNATURAL AMINO ACIDS" dated October 31, 2002

WO02/086075 "METHODS AND COMPOSITIONS FOR THE PRODUCTION OF ORTHOGONAL tRNA-AMINOACYL tRNA SYNTHETASE PAIRS" dated October 31, 2002.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above can be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.

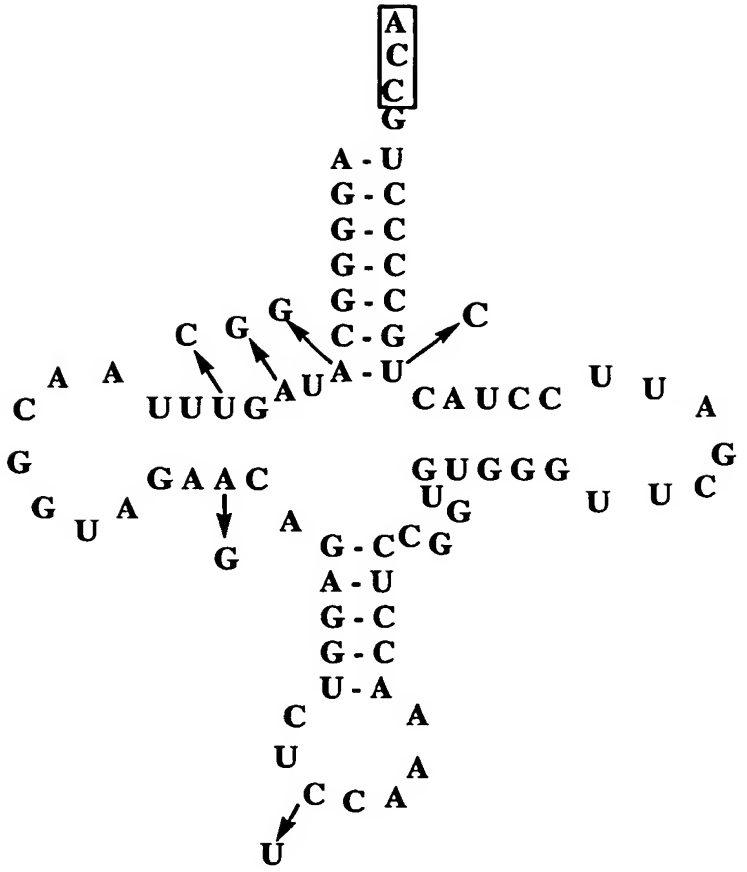
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Figure 1.

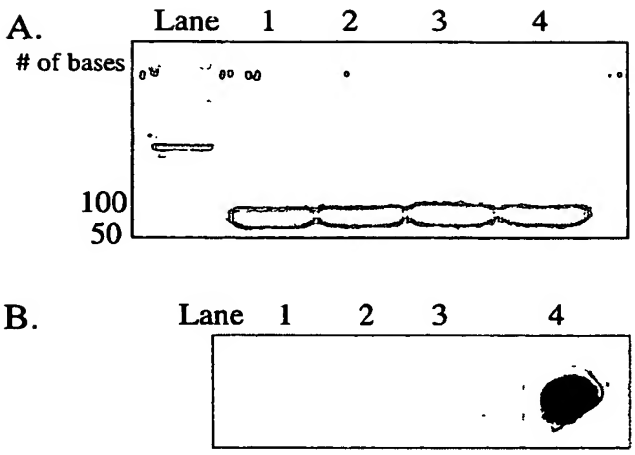


Figure 2.

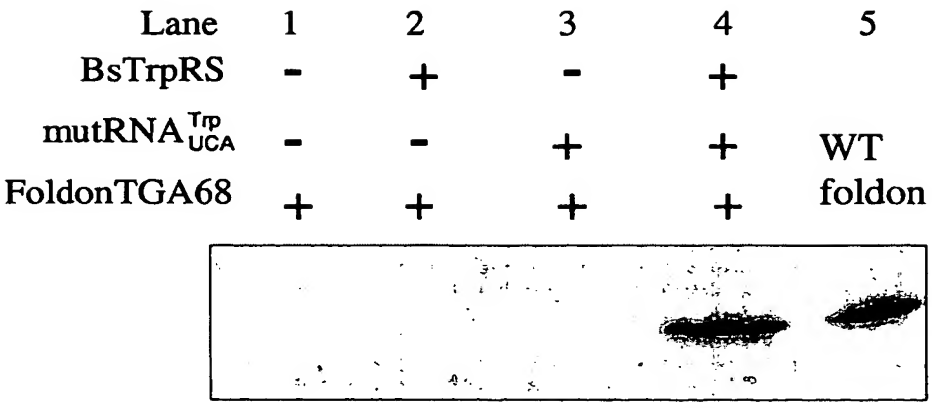


Figure 3.

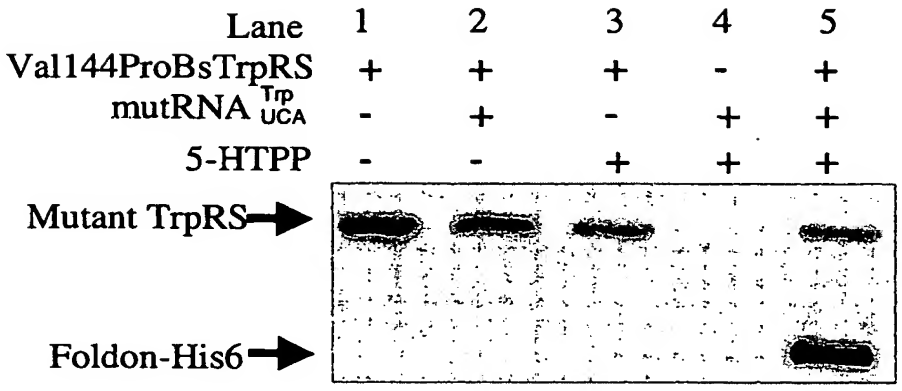
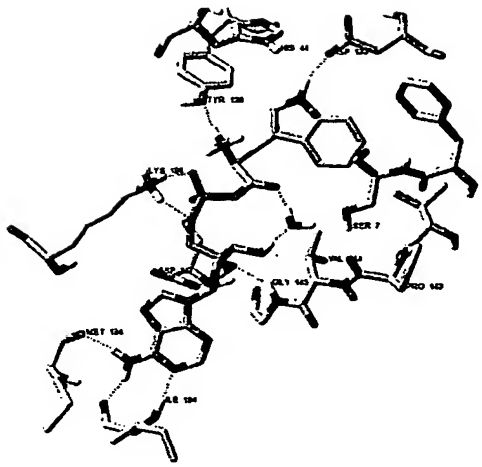
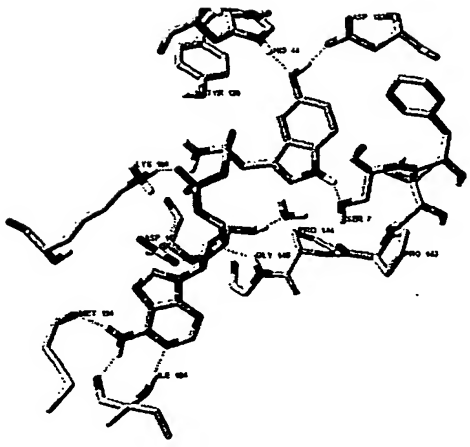


Figure 4.



Wild type BsTrpRS•Trp-5'AMP complex



Val144ProBsTrpRS•5HTPP-5'AMP complex

Figure 5.

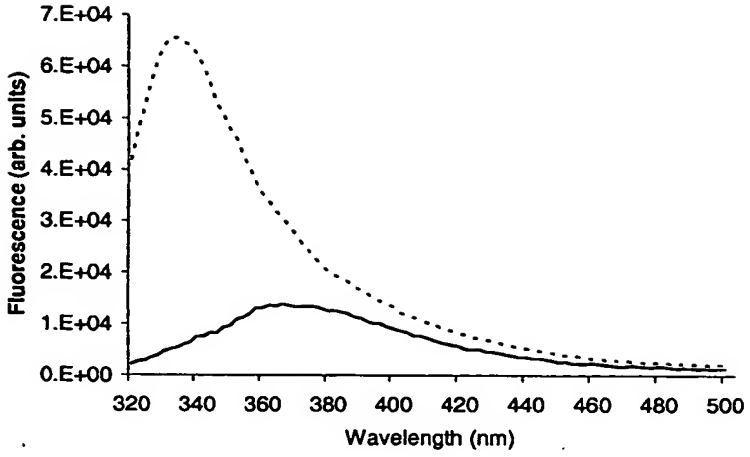
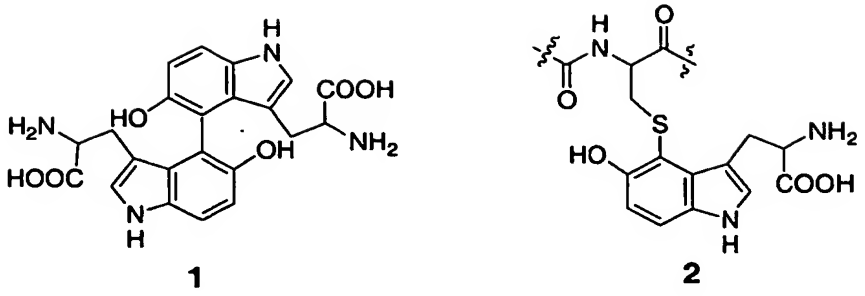

7.E+04
6.E+04
5.E+04
4.E+04
3.E+04
2.E+04
1.E+04
0.E+00
Fluorescence (arb. units)
320
340
360
380
400
420
440
460
480
500
Wavelength (nm)

Figure 6.

7A.



7B.

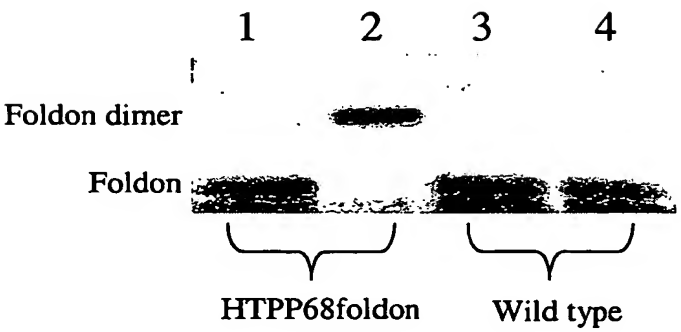


Figure 7.

Application Information

Application Type::	Provisional
Subject Matter::	
Suggested Classification::	
Suggested Group Art Unit ::	
CD-ROM or CD-R?::	
Number of CD disks::	
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Sequence submission::	*CD or Paper*
Computer Readable Form (CRF)?::	
Number of copies of CRF::	
Title Line One::	SELECTIVE INCORPORATION OF 5-
Title Line Two::	HYROXYTRYPTOPHAN INTO PROTEINS IN
Title Line Three::	MAMMALIAN CELLS
Attorney Docket Number::	54A-001000US
Request for Early Publication?::	
Request for Non—Publication?::	
Suggested Drawing Figure::	
Total Drawing Sheets::	7
Small Entity::	No
Petition included?::	
Petition Type::	
Secrecy Order in Parent Appl.?::	

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